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Inhibition by active site directed covalent modification of human glyoxalase I

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ABSTRACT

The glyoxalase pathway is responsible for conversion of cytotoxic methylglyoxal (MG) to D-lactate. MG toxicity arises from its ability to form advanced glycation end products (AGEs) on proteins, lipids and DNA. Studies have shown that inhibitors of glyoxalase I (GLO1), the first enzyme of this pathway, have chemotherapeutic effects both in vitro and in vivo, presumably by increasing intracellular MG concentrations leading to apoptosis and cell death. Here, we present the first molecular inhibitor, 4-bromoacetoxy-1-(*S*-glutathionyl)-acetoxy butane (4BAB), able to covalently bind to the free sulfhydryl group of Cys60 in the hydrophobic binding pocket adjacent to the enzyme active site and partially inactivate the enzyme. Our data suggests that partial inactivation of homodimeric GLO1 is due to the modification at only one of the enzymatic active sites. Although this molecule may have limited use pharmacologically, it may serve as an important template for the development of new GLO1 inhibitors that may combine this strategy with ones already reported for high affinity GLO1 inhibitors, potentially improving potency and specificity.

1. Introduction

The cellular glyoxalase system detoxifies cytosolic reactive aldehydes that occur with metabolism. It is composed of the enzymes glyoxalase I (GLO1, homodimer)[1,2] and glyoxalase II (GLO2, monomer)[3] and is responsible for the conversion of the methylglyoxal (MG) to D-lactate via the intermediate *S*-D-lactoylglutathione using catalytic amounts of glutathione (GSH, Scheme 1).[4] MG is a small dicarbonyl that arises primarily through the non-catalytic breakdown of triosephosphates (i.e., dihydroxyacetone phosphate and glyceraldehyde-3-phosphate)[5,6] and from the spontaneous breakdown of glucose.[7] MG has the ability to form advanced glycation end-products (AGEs) on proteins,[8,9] lipids[10] and DNA,[11] resulting in detrimental effects in aging,[12] as well in diseases such as diabetes[13–15] and Alzheimer's disease,[16,17] where GLO1 protein expression and activity have been shown to be reduced. GLO1 and MG have also been linked to behavioral conditions such as anxiety[18–20] and depression.[20] Distler et al. identified MG as a $GABA_A$ receptor agonist and reported that overexpression of GLO1 increases anxiety by reducing levels of MG[19] and pretreatment with MG or inhibition of GLO1 reduced pharmacologically-induced seizures in mice.[21]

Additionally, increased GLO1 gene expression, protein expression, and activity have been reported in a variety of cancers, including breast,[22–24] pancreatic,[25] melanoma,[26] and prostate.[27–29] Vince and Daluge proposed that inhibitors of GLO1 could serve as antitumor agents, by increasing concentrations of MG in tumor cells.[30] S-substituted glutathione derivatives have been shown to be competitive inhibitors of GLO1, with *S*-*p*-bromobenzylglutathione being the most potent of these inhibitors (K_i = 0.08 μM).[31] Thornalley et al.[32] and Sakamoto et al.[33] reported that *S*-*p*-bromobenzylglutathione diesters had antitumor activity in human leukemia 60 cells and human lung cancer cells, respectively. Murthy et al. reported the first transition state analogues of GLO1 with K_i values ranging from 14 to 160 nM[34] and Sharkey et al. reported that the diethyl ester prodrug of *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)glutathione (CHG) was able to inhibit tumor growth in mice.[35] More recently, a variety of GLO1 inhibitors have been synthesized, including the first bivalent-transition state analogues of GLO1 (K_i 0.96–84 nM),[36] in which two CHG molecules are covalently linked together, enhancing the effective concentration of inhibitor at the second active site. Additionally, More and Vince

Abbreviations: CHG, *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)glutathione; AGE, advanced glycation end product; MG, methylglyoxal; GSH, reduced glutathione; GLO1, glyoxalase I.

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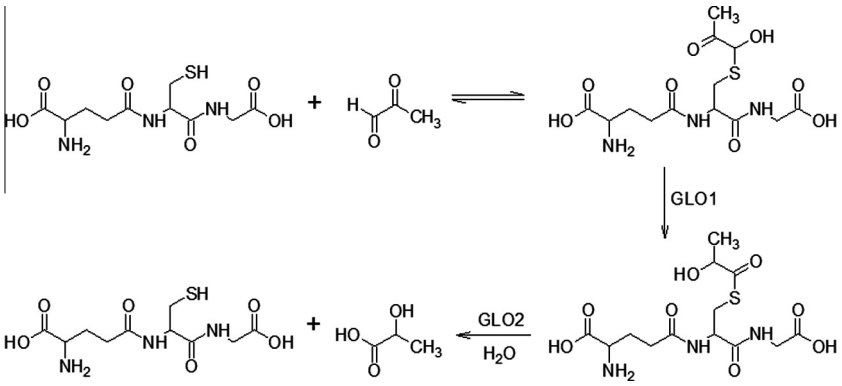

Scheme 1. Overview of the glyoxalase system.

have reported GLO1 inhibitors with K_i values in the low micro/nano-molar range[37,38] that are metabolically stable and resistant to γ-glutamyltranspeptidase, which is capable of cleaving the γ-Glu-Cys bond of compounds such as *S*-*p*-benzoylglutathione and the *S*-(*N*-aryl-*N*-hydroxycarbamoyl)glutathione derivatives.[39,40]

To date, all of the GLO1 inhibitors above have been either substrate or transition state analogues, and while some of these molecules show strong potency, the potential to increase potency and specificity should not be ignored. To directly address specificity, we have taken a novel approach in designing an active-site directed covalent inhibitor of GLO1. The covalent inhibitor was designed based on the X-ray crystallographic structures of the dimeric GLO1 with the substrate analogue *S*-*p*-bromobenzyl glutathione[2] and the transition state analogue *S*-(*N*-4-iodophenyl-*N*-hydroxycarbamoyl)glutathione.[1] These 3-dimensional structures indicate that there is a hydrophobic binding pocket composed of residues from both monomers (Met157, Leu160, Phe162, Leu174, Met179, Met183 from one monomer; Cys60, Phe62, Met65, Phe67, Leu69, Phe71, Ile88 from the other) adjacent to the enzyme active site, with the free sulfhydryl group of C60 sticking into this hydrophobic pocket.

The goal of this study is to provide a proof of principle that GLO1 could be inhibited covalently at Cys60 within the enzyme active site and the aims of this study were threefold: (1) to synthesize a rudimentary GSH-analogue with a leaving group that could potentially modify GLO1 covalently, (2) characterize the kinetic parameters of this compound, and (3) determine the amino acid modified in the active site. Here we report the inhibitor 4-bromoacetoxy-1-(*S*-glutathionyl)-acetoxy butane (4BAB, Fig. 1) that is able to covalently modify Cys60 in the enzyme active site of GLO1.

2. Experimental

2.1. Materials and reagents

LC/MS solvents were obtained from Burdick and Jackson and were of highest purity possible. Recombinant human GLO1 was expressed and purified according to published methods and is briefly described below.[41] All other reagents were of the highest grade possible.

2.2. Synthesis of 4-bromoacetoxy-1-(*S*-glutathionyl)-acetoxy butane (4BAB)

The synthetic route to 4BAB is outlined in Figure 1. First, the starting di-ester bromoacetic acid 4-(2-bromo-acetoxy) butyl ester (BBE) was synthesized by dissolving 1.00 g (1.02 mL, 0.011 mol) of 1,4-butanediol in 50 mL of methylene chloride followed by addition of 4.44 g (1.92 mL, 0.022 mol) of bromoacetyl bromide, producing a yellow transparent solution. The solution was refluxed for 20 h and the solvent was removed in vacuo using a Buchi Rotavapor Re111 Evaporator yielding an orange tinted crystalline solid. The crystals were re-dissolved in a minimum amount of methanol and re-crystallized by the addition of water slowly until a white precipitate formed. The precipitate was vacuum filtered and air dried overnight. The crystals were a flaky white solid with an 82% percent yield and melting point of 60–62.5 °C. The ^{1}H NMR was consistent with the structure of BBE (δ 1.75–1.79 (t, –O–CH_2CH_2–); δ 3.83 (s, Br–CH_2C(O)O–); δ 4.19–4.23 (t, –C(O)OCH_2–), Fig. S1). Four grams (12 mmol) of BBE was dissolved in 150 mL of methanol with stirring. A glutathione (GSH) solution was prepared by dissolving 0.8329 g (2.71 mmol)

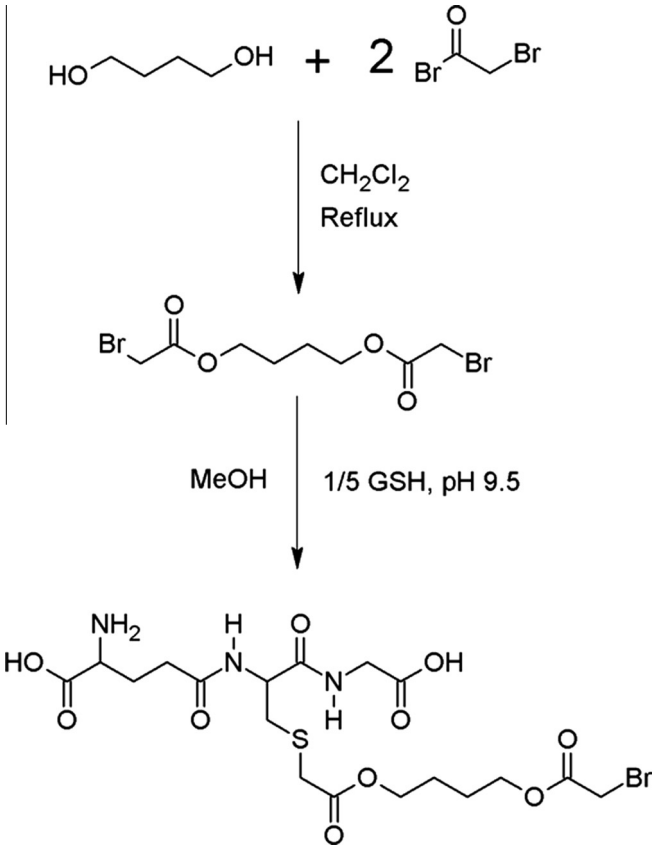

Figure 1. Synthetic route to the GLO1 covalent inhibitor 4BAB.

of GSH in 15 mL of degassed water and treating this solution with 1.1 mL of 5 M NaOH to bring the pH of the solution to ~9.5. The GSH solution was added all at once to the BBE solution with vigorous stirring. After two minutes, formic acid was added until the pH of the solution was ~3.5. The solvent was removed in vacuo and a white solid remained. Water, 20 mL, was added to the solid to dissolve 4BAB and any water-soluble impurities and 150 mL of diethyl ether was added to dissolve the excess BBE. The two layers from the filtrate were separated and the aqueous layer was saved and washed once more with 150 mL of ether. The aqueous layer was filtered through an aqueous syringe filter tip and 4BAB was purified by on a Waters Delta 600 HPLC equipped with a Waters 996 Photodiode Array Detector with a SymmetryPrep™ C18 (7 μm 100 Å 19 × 150 mm) column (Waters) using a linear AB gradient from 5% to 60% B for 12.5 min and 60% to 100% B for 2.5 min and a flow rate of 10 mL/min (where Solvent A was 0.1% aqueous TFA and Solvent B was 0.1% TFA in acetonitrile). The major peak at 9 min was collected and the product concentrated until ~2 mL remained. This was flash frozen in an acetone/dry ice bath for 15 min then lyophilized to dryness. Clear/white crystals remained with a percent yield of 20%. The ^{1}H NMR was acquired on a ECX 400 MHz NMR (JEOL, Peabody, MA, USA) and MS analysis was performed on a Apex IV Qh Ultra FT-ICR (Bruker Daltonics, Fremont, CA, USA). Both the ^{1}H NMR (δ 1.76–1.80 (m, –O–CH$_2$CH_2–); δ 2.19–2.28 (m, Glu-C$_\beta H_2$); δ 2.56–2.63 (m, Glu-C$_\gamma H_2$); δ 2.93–2.99 (q, Cys-C$_\alpha H_a$); δ 3.12–3.17 (q, Cys-C$_\alpha H_b$); δ 3.41–3.49 (q, –S–CH_2–C(O)); δ 4.02 (s, –OC(O)–CH_2–Br), δ 4.05 (s, Gly-CH_2); 4.05–4.09 (t, Glu-C$_\alpha H$); δ 4.21–4.27 (m, C(O)O–CH$_2$–CH_2–); δ 4.59–4.63 (q, Cys-C$_\alpha H$) and MS analysis are consistent with the structure and theoretical mass of 4BAB (Supplemental Figs. S2–S4, respectively).

2.3. Purification of human GLO1 enzyme

Human GLO1 clone[41] in a pKK223-3 expression vector was obtained as a gift from Prof. Bengt Mannervik at the University of Uppsala, Sweden. The plasmid was introduced into *Escherichia coli* JM103 cells as previously described using electroporation (2.5 kv, 25 μF, and 400 Ω).[42] Cells were plated on Luria–Bertani (LB) agarose plates supplemented with 100 μg/mL of ampicillin to screen for colonies that had been transfected with the plasmid. To ensure that the positively transfected cells contained the correct GLO1 coding sequence, the plasmid of a selected single colony was extracted using the Spin Miniprep Kit (Qiagen, Hilden, Germany) and sequenced with a primer having sequence 5′-GCAC TACA TTAA GGTT GCCA TTTT GTTA GG-3′. The recombinant enzyme was overexpressed, purified, and quantified (ε_{280} = 1.67 mL mg^{-1} cm^{-1}) according to published procedures.[41] Briefly, a single colony was cultured in LB broth at 37 °C until a cell density of reached $A_{600} \approx 0.3$; the expression of GLO1 was induced for 12 h at 30 °C by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside and 1.0 mM zinc chloride. The bacteria were lysed using a French Press, and the GLO1 protein was purified from the supernatant using *S*-hexylglutathione agarose affinity chromatography as previously described[41] followed by gel-filtration chromatography (Sephadex G-50). The mass and purity of the enzyme were assessed using both SDS and native polyacrylamide gel electrophoresis. SDS–PAGE (4–12% Bis–Tris) resolved two bands with molecular weights of approximately 21 and 42 kDa, which are consistent with the amino acid deduced monomer (20,647 Da) and dimer (41,294 Da) masses of GLO1, respectively (Fig. S5a). Native PAGE (4–16% Bis–Tris) showed one band consistent with the MW for the GLO1 dimer (41,294 Da) confirming that only the dimer is present under native conditions (Fig. S5b). ESI-MS analysis of the purified protein showed multiply charged species that were consistent with a MW of 20,647 Da for the enzyme monomer.

2.4. Purification and esterification of GLO1 carboxymethylated peptide C_{60}-D-F-P-I-M-K_{66}

The peak corresponding to the carboxymethyl modified peptide 60–66 (C_{60}-K_{66}CM, *m*/*z* 911.4, resulting from hydrolysis of the ester bond of the 4BAB) from the GLO1:4BAB trypsin digest solution (detailed below) was purified by LC–MS (details in Supplemental methods) and the C_{60}-K_{66}CM peptide collected was used as a stock solution (the concentration of the peptide was not determined). Esterification of the C_{60}-K_{66}CM peptide was carried out by treatment of 10 μL C_{60}-K_{66}CM with 2 μL of concentrated sulfuric acid followed by 68 μL of either water (control), 95% ethanol, or *n*-propanol and incubated at room temperature for 16 h, at which point 20 μL of 1 M formic acid was added to the mixture and the samples were analyzed by LC–MS (details in Supplemental methods).

2.5. Activity assays

2.5.1. Inactivation and competitive inhibition of GLO1 by 4BAB

Inactivation of GLO1 by 4BAB was carried out at concentrations of 4.7, 14.4, 31.0 and 80.6 μM in 75 mM HEPES, pH 7.3 and were incubated with GLO1 at 25 °C ranging from 5 to 180 s after which point GLO1 activity was measured as previously described.[43] Enzymatic activities are reported as the initial rate of the reaction measured by the change in OD$_{240}$/min using a DU640 spectrometer (Beckman Coulter, Brea, CA). The concentration of thiohemiacetal substrate used in the reaction was 0.18 mM[44] and the concentration of free GSH was fixed at 0.20 mM for all assay solutions. Substrate mixtures were allowed to equilibrate for 30 min in 50 mM HEPES, pH 7.3 at 25 °C in a 1 cm path length cuvette with a final total volume of 1 mL, after which point 100 μL of enzyme inactivation solution was added to the cuvette to measure activity. After the addition of enzyme, the initial rate (v_0) of the enzyme catalyzed reaction was obtained from the increase in absorbance at 240 nm over one minute due to the formation of *S*-D-lactoylglutathione (ε_{240} = 2860 M^{-1} cm^{-1}).[43]

$$v_0\ (\mu\text{mol min}^{-1}) = \frac{(dA_{240}/dt)\ \text{min}^{-1} \times 1000\ \mu\text{L}}{\varepsilon_{240}\ \text{M}^{-1}\ \text{cm}^{-1} \times 1.0\ \text{cm}}$$

The maximum velocity (V_{max}) was subsequently calculated from the Michaelis–Menten equation using K_m = 0.18 mM for GLO1.[44] Plots of ln(v_n/v_0) versus time (where v_n is the rate at $t = n$ and v_0 is rate at $t = 0$, Fig. 2A) and Abs240 versus time (Fig. S6a) were constructed to determine the extent of inactivation. For 4BAB competitive inhibition studies, GLO1 activity was measured as described above at varying substrate concentrations (0.10–0.70 mM) in the presence of 0.00–157.6 μM 4BAB.

2.5.2. Protection of GLO1 against 4BAB inactivation

GLO1 was incubated in 75 mM HEPES pH 7.3 at 25 °C with 13.1 μM 4BAB in the presence of the competitive inhibitor CHG at concentrations of 0.00 μM, 0.108 μM and 0.324 μM. The activity was measured and plots of ln(v_n/v_0) versus time (Fig. 2D) and Abs240 versus time (Fig. S6b) were constructed as described above to determine the extent of inactivation.

2.5.3. Kinetic analysis of GLO1:4BAB complex

A solution of GLO1:4BAB was made by mixing 100 μL of 2.3 mg/mL GLO1 stock with 500 μL 100 mM HEPES pH 7.4, 300 μL H_2O and 100 μL 13.45 mM 4BAB (final concentrations: 50 mM HEPES pH 7.4, 0.3 mg/mL GLO1, and 0.672 mM 4BAB). The activity of the solution was monitored until there was no change in activity over time. After 5 min, the activity stabilized and the reaction was treated with 2 mL of H_2O and washed using a Millipore Centriplus Filter (MW cutoff of 10,000 Da) at 3000 g for 45 min at 4 °C to remove any unreacted 4BAB, with the

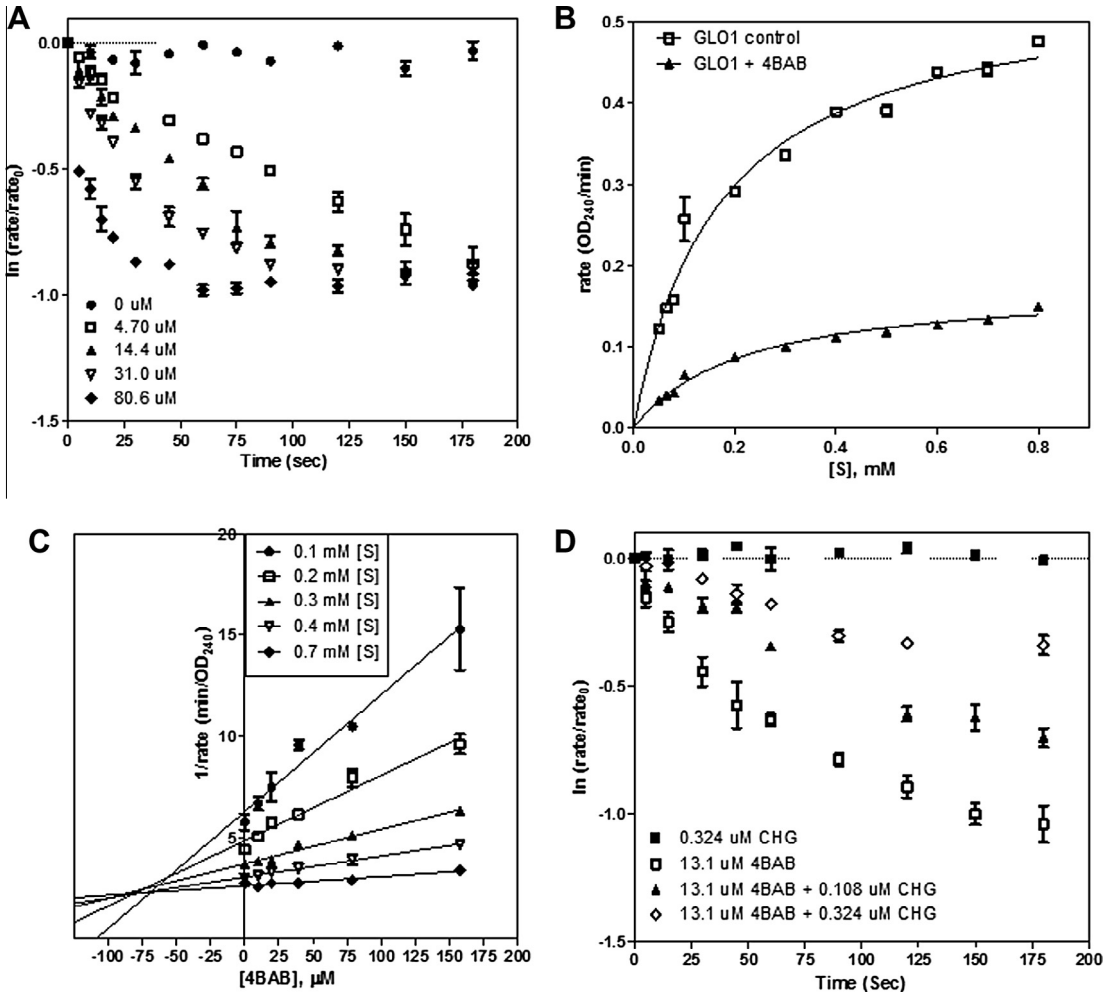

Figure 2. Kinetic characterization of 4BAB on GLO1. (A) GLO1 inactivation by 4BAB expressed as % remaining activity versus time. (B) Michaelis–Menten kinetics for GLO1 control and GLO1:4BAB solutions. Kinetic constants are given in Table 1. (C) Dixon plot showing competitive inhibition of GLO1 by 4BAB. (D) Protection against inactivation of GLO1 by 4BAB using the strong competitive inhibitor CHG (K_i = 46 nM).

subsequent addition of 2 mL of H_2O and washed under the same conditions. A control solution (GLO1 control) was prepared in the same manner, substituting 100 μL of water for 4BAB and enzyme concentrations were determined by measuring the absorbance at 280 nm using ε_{280} 1.67 mL mg^{-1} cm^{-1} for GLO1.[41] The starting enzyme concentrations were 1.24 and 1.20 μg/mL for the GLO1 control and GLO1:4BAB solution, respectively. Activities at various substrate concentrations (0.05–0.8 mM thiohemiacetal) were measured in the presence of 50 mM HEPES pH 7.4 using 100 μL of the working enzyme solution.

2.6. Sample preparation and LC–MS analysis of intact and trypsin digested GLO1:4BAB complex

The GLO1 control and GLO1:4BAB solutions prepared for the kinetic analysis above were analyzed by LC–MS with and without tryptic digestion. For each trypsin digestion, 10 μL of GLO1 control and GLO1:4BAB solutions were treated with 90 μL of 100 mM HEPES pH 7.3 followed by 300 μL of digestion solution containing 6 M urea, 2 mM DTT, 50 mM NH_4HCO_3 pH 8.05 and incubated at 95 °C for 30 min. The solution was cooled to room temperature and treated with 300 μL of dilution buffer containing 50 mM NH_4HCO_3 pH 8.05, 1 mM $CaCl_2$ and 4 μL of 0.5 μg/μL trypsin and incubated at 37 °C for 4 h. Three additional 4 μL portions of trypsin were added over a total incubation time of 29 h. The digestion was stopped by addition of 10 μL 50% TFA and 25 μL TCEP and flash freezing the solution in an acetone/dry ice bath. Tryptic peptides were analyzed by LC–MS/MS (details in Supplemental methods).

3. Results

3.1. Synthesis of BBE and 4BAB

Synthesis of BBE and 4BAB was confirmed by 400 MHz ^{1}H NMR (Figs. S1 and S2) and 4BAB was confirmed by high resolution MS analysis (Figs. S3 and S4). Proton NMR peaks for 4BAB corresponding to those of the glutathione moiety were assigned as previously reported.[45] The remaining peaks corresponding to 4BAB were assigned based on the ^{1}H NMR of BBE. ESI-MS was consistent with the singly charged monoisotopic mass of 4BAB (theoretical monoisotopic singly charged m/z = 558.07515, observed m/z = 558.09332) and the spectrum showed the splitting due to the two isotopes (Br^{79} and Br^{81}) of bromine (Fig. S4), which are approximately equally abundant in nature.

3.2. Inactivation/covalent modification of GLO1 by 4BAB

Human GLO1 was inactivated by 4BAB (Figs. 2A and S6a), although activity of GLO1 never reached zero. MS analysis of the

GLO1:4BAB complex (Fig. S6) showed two peaks, the first peak consistent with the mass of the GLO1 monomer modified by 4BAB (21,125 Da) and the second peak consistent with the unmodified monomer mass (20,647 Da). A comparison of the kinetics of substrate turnover and binding by 4BAB modified and unmodified GLO1 was carried out (Fig. 2B, Table 1). Data for the competitive inhibition of GLO1 by 4BAB is illustrated in Fig. 2C. The effect on 4BAB inactivation of GLO1 by the addition of the transition state analogue CHG (K_i = 46 nM)[34] is indicated in Figs. 2D and S6b. The interaction of 4BAB was confirmed to be covalent by LC–MS analysis of the GLO1:4BAB complex (Fig. S7), as there is a peak present with m/z values consistent with the GLO1 monomer modified by 4BAB (minus the bromine, which has been displaced) (Table S1). The modification was localized to the tryptic peptide comprising the amino acid sequence C_{60}-K_{66} as evident by the reduction in ion intensity for the C_{60}-K_{66} peptide (singly charged m/z = 853.5, Fig. 3A and B) and appearance of a new ion species was observed for the GLO1:4BAB complex (singly charged m/z = 911.4, Fig. 3C and D). Tandem MS analysis and carboxylic acid side chain esterification of the C_{60}-K_{66} peptide confirmed Cys60 to be the site of modification (Figs. 4 and 5).

4. Discussion

Although the synthesis of 4BAB is straightforward, the final yields of 4BAB only ranged from 9% to 25%. The reason for the low yields is not entirely clear, but possible sources of product loss could be the breakdown of product during washes, formation of the di-GSH substituted diester, or loss of product during purification. The starting dibromo-diester is in 5-fold excess over GSH, which should prevent, or at least limit, the formation of the di-GSH substituted diester. However, ^{1}H NMR of the HPLC peak preceding the product peak (data not shown) shows that this side product is formed in the reaction mixture, which in turn decreases

Table 1
Kinetic constants for GLO1 control and the GLO1:4BAB complex. Values are given as the mean ± S.D

Enzyme	Specific activity (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM $^{-1}$)
GLO1	1580 ± 52	1088 ± 36	182 ± 18	5.98 ± 0.62
GLO1:4BAB	516 ± 22	355 ± 15	221 ± 26	1.61 ± 0.21

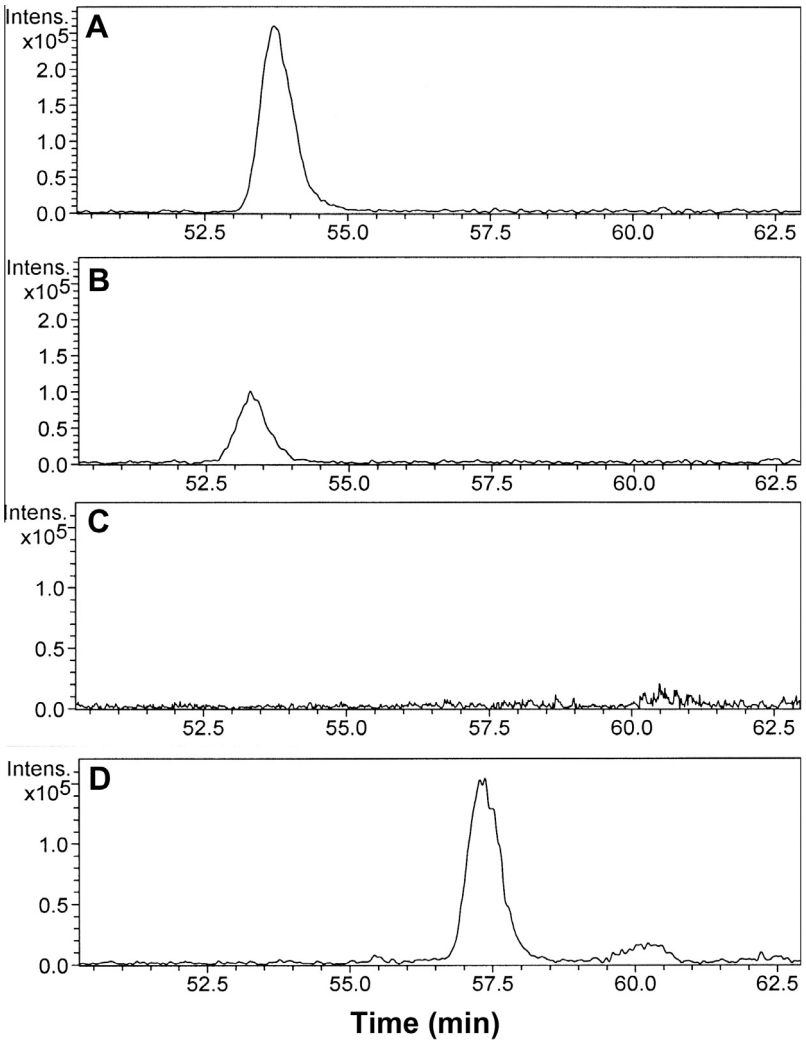

Figure 3. MS analysis of 4BAB interaction with GLO1. Extracted ion currents (XICs) for ion 853.5 ± 0.5 Da for (A) GLO1 control and (B) GLO1:4BAB trypsin digests as well as XICs for 911.4 ± 0.5 Da for (C) GLO1 control and (D) GLO1:4BAB trypsin digests.

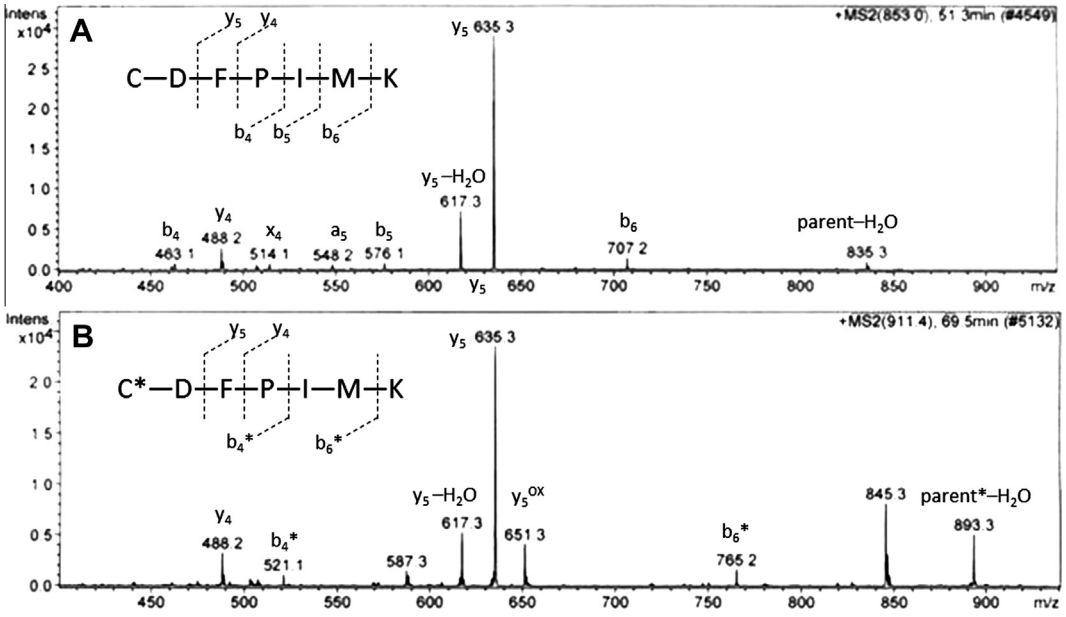

Figure 4. Collision induced dissociation of C_{60}-K_{66} and C_{60}-K_{66}CM peptides. (A) Fragmentation of parent ion 853.4 *m*/*z* eluting at 53 min after trypsin digestion of GLO1, consistent with peptide C_{60}-K_{66}. The parent ion and all fragment ions are singly charged. (B) Fragmentation of parent ion 911.4 *m*/*z* eluting at 57 min from trypsin digestion of GLO1:4BAB. The 911.4 *m*/*z* is consistent with modification of C_{60}-K_{66} by a carboxymethyl group and the asterisk indicates carboxymethyl modification. The parent ion and all fragment ions are singly charged and the fragmentation pattern limits the site of modification to either Cys60 or Asp61.

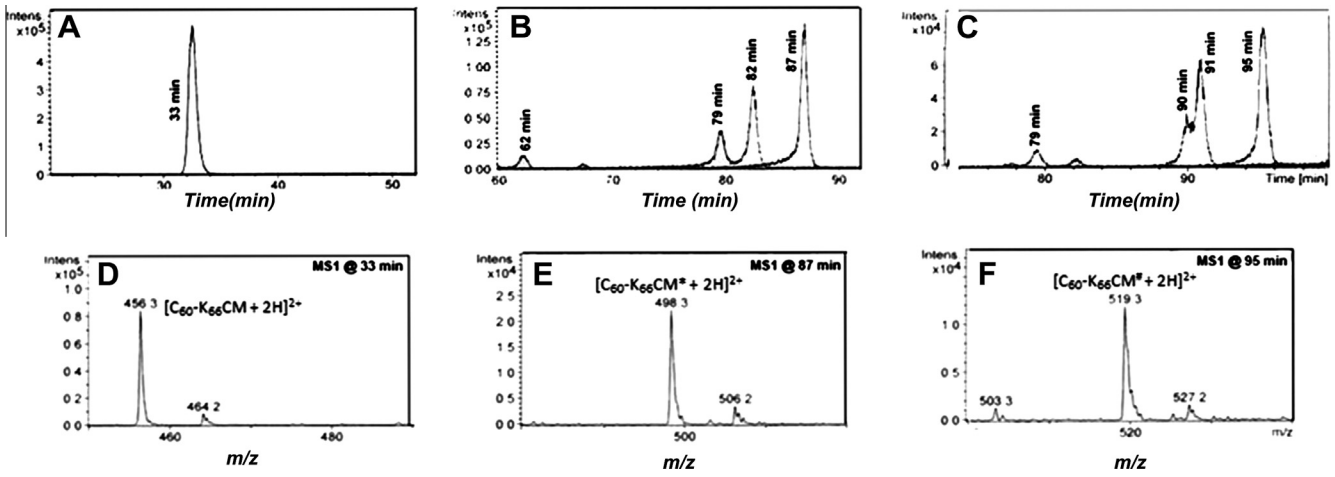

Figure 5. LC/MS analysis of the C_{60}-K_{66}CM control, ethanol, and propanol esterification reactions. The TIC chromatogram for the control (A) shows a single peak with m/z consistent with doubly charged C_{60}-K_{66}CM peptide (D), $[C_{60}\text{-}K_{66}CM+2H]^{2+}$ = 456.3 *m*/*z*. TIC chromatograms for the ethanol (B) and propanol (C) esterification of C_{60}-K_{66}CM. The first peak in B and C corresponds to the mono-esterified C_{60}-K_{66}CM peptide, the second and third peaks correspond to variants of the di-esterified C_{60}-K_{66}CM peptide, and the last peak corresponds to the tri-ethyl esterified C_{60}-K_{66}CM peptide (E, C_{60}-$K_{66}CM^{*}$) and tri-propyl esterified C_{60}-K_{66}CM peptide (F, C_{60}-$K_{66}CM^{\#}$). Full chromatograms and MS spectra can be found in the supplemental material.

the overall yield of desired product. Additionally, it is possible that the leaving group is hydrolyzed during the wash cycles. The pH of the GSH solution used in the reaction was ~9.5 with the reaction stopped by the addition of formic acid until the pH is 3.5. This should prevent the hydrolysis of the leaving group, although the low pH could make the ester bonds susceptible to hydrolysis. There is also the possibility of other side reactions that have not been accounted for here.

The kinetic data obtained from incubation of 4BAB with GLO1 show that 4BAB does not completely inactivate the enzyme (Figs. 2A and S6a). At low concentrations (4.7 and 14.4 μM), 4BAB seemed to follow biphasic kinetics (Fig. 2A); even though regardless of the concentration 4BAB, there was a plateau at approximately the same level of maximal activity. This was quite unexpected as we had proposed that 4BAB enters the enzyme active site and orients the bromine near Cys60 for nucleophilic attack, which should completely abolished enzyme activity. This led to the question of how 4BAB could be covalently modifying GLO1 but result in partial enzyme inactivation. One explanation was that 4BAB could possibly be binding to GLO1 somewhere other than the enzyme active site, which results in partial loss of enzyme activity. However, this does not seem likely, since it was shown that the transition state analogue CHG protects GLO1 from inactivation by 4BAB (Fig. 2D), which suggests that inactivation is an active site directed process. Therefore, another molecular phenomenon must be causing the observed partial inactivation.

Another possibility is that there is active GLO1 monomer present in the assay solution, and it is this species that inactivated by 4BAB leading to the observed activity decrease. However this most

likely is not the case since the native PAGE shows that GLO1 (Fig. S5b) is present exclusively as the dimer. Furthermore, there are no reports to our knowledge of monomeric human GLO1 retaining enzyme activity. This led to the hypothesis that only one active site of the GLO1 homodimer is being modified by 4BAB. To test this hypothesis, GLO1 treated with 4BAB was analyzed by LC–MS and two species were observed in the reaction mixture; one that corresponded to the unmodified GLO1 monomer and one that corresponded to GLO1 monomer with 4BAB (minus the bromine) attached. Furthermore, the two species are present in nearly equal quantities, although the peaks were not sufficiently resolved to integrate or quantify (Fig. S7). If there was only one active site of GLO1 modified, then it would be expected to maintain 50% of its activity but only 33% activity remains. We proposed that modification of one active site of GLO1 could cause a conformational change affecting the other active site present in the dimeric form. The kinetic constants were measured for GLO1 control and for the GLO1:4BAB complex (Fig. 2B, Table 1) and the k_{cat} and K_m values obtained for GLO1 are in good agreement with values previously reported for the enzyme.[44] The GLO1:4BAB enzyme complex showed reduced catalytic efficiency such that there was a 2/3 loss of catalytic turnover (decreased k_{cat}) and about 1/5 decrease in substrate binding (increased K_m) for the GLO1:4BAB enzyme complex compared to the wild-type enzyme. This is consistent with the observation that GLO1 retains about 33% activity in the presence of 4BAB (Fig. 2A). A scenario could be envisioned in which one molecule of 4BAB binds covalently to a single active site resulting in complete inactivation simultaneously causing a conformational change around the opening to the hydrophobic pocket of second active site that results in the reduced kinetic parameters observed. This conformational change could presumably prevent a second molecule of 4BAB from orienting the bromine group near Cys60 of the second active site for attack. The inaccessibility to the hydrophobic pocket should not have that much of an effect on the binding of the thiohemiacetal substrate (about 20% decrease observed), since the methyl group of substrate is much smaller than the tail region of 4BAB. The proposal that modification at one active site affects the binding at the second active site seems contradictory to the results reported for bivalent transition state analogues,[36] which have a lower K_i than the transition state molecules by themselves. However, these bivalent transition state analogues bind to the active site in the same manner as a single transition state molecule, which is by coordination of the *N*-hydroxycarbamoyl moiety of the molecule to the active site zinc ion. There has yet to be evidence that indicates that GLO1 has cooperative binding with regards to substrate or transition state analogues.[34,41] Allosteric coupling has been reported for the two active sites of the monomeric *Plasmodium falciparum* GLO1[46] and was proposed for the two active sites of monomeric yeast GLO1[47] but no reports of allosteric binding have been reported for the dimeric human enzyme.

Given that inactivation of GLO1 by 4BAB is an active site directed mechanism, as the transition state analogue CHG protects GLO1 against inactivation by 4BAB (Figs. 2D and S6b), we next set out to determine the amino acid that is covalently modified by 4BAB. From the X-ray crystallographic structures[1,2] Cys60 is located in the hydrophobic binding pocket and is the most likely candidate to act as the nucleophile to displace the bromine on 4BAB. To test this hypothesis, the GLO1:4BAB complex was digested with trypsin and analyzed by LC–MS/MS and based on the in silico tryptic peptides predicted for GLO1, Cys60 would lie on a 7 amino acid long peptide (C60-D-F-P-I-M-K66, C_{60}-K_{66}). Complete disappearance of the 853.4 *m*/*z* ion (corresponding to the singly charged C_{60}-K_{66} peptide) was not observed, consistent with incomplete inactivation of the enzyme, leaving a fraction of the Cys60 residues unmodified. There should also be a new peak present in the chromatograms for the GLO1:4BAB trypsin solution, corresponding to a modified C_{60}-K_{66} peptide. Modification of the C_{60}-K_{66} peptide by 4BAB should produce a singly charged *m*/*z* value of 1330.5. However, no new peaks corresponding to the singly or multiply charged ions consistent with this modification were present in the GLO1:4BAB trypsin digests.

Unexpectedly, the intact inactivator was not observed on the C_{60}-K_{66} peptide, and therefore we set out to explore the possible reasons for the absence of the modified C_{60}-K_{66} peptide in the chromatograms. One possible explanation could be a missed cleavage at Lys59 resulting from increased steric hindrance around this residue, possibly making it inaccessible to trypsin, but this does not seem to be the case since there is no ion present in the chromatographs consistent with modification of the V_{51}-K_{66} peptide. Another possible explanation could be that the ester functions of the bound 4BAB are hydrolyzed during trypsin digestion, as trypsin has been shown to possess esterase activity towards benzoyl-L-arginine ethyl ester (BAEE) and other L-arginine esters.[48,49] We predicted the possible species (Table S2) that would arise from hydrolysis of these esters, assuming that C_{60}-K_{66} is the peptide modified, and checked the chromatograms for the existence of these ions. Monitoring the extracted ion currents of these ions showed the presence of an additional peak in the GLO1:4BAB tryptic digest with an elution time of 57 min that was not present in the control mixture (Fig. 3C), and this new peak was due to the presence of a singly charged ion with *m*/*z* of 911.4 (Fig. 3D). This mass is consistent with the C_{60}-K_{66} peptide plus the addition of a carboxymethyl group (58 mass units), one of the predicted species resulting from the hydrolysis of the ester bonds of 4BAB (C_{60}-K_{66}CM, Table S2). LC–MS/MS analysis confirmed that the modification was localized to either Cys60 or Asp61, both of which could act as nucleophiles to displace the bromine. First, the mass increase of 58 units of the b_4 ion in Fig. 4B indicates that the modification must be somewhere on peptide fragment C_{60}-D-F-P_{63}. Second, the fact that the y_5 fragment is unchanged in Figure 4B means that residues F_{62}-P-I-M-K_{66} are excluded as possibilities. This leaves only residues Cys60 and Asp61 as the possible sites of modification. Since low mass fragments in the MS2 spectra were not detected, localization of the modification site had to be achieved through alternative methods.

The presence of a carboxymethyl group on either Cys60 or Asp61 would yield two structurally different peptides with similar but different chemical properties (referred to as C_{60}-K_{66}CMCys if modification is at Cys60 and C_{60}-K_{66}CMAsp if modification is at Asp61, Fig. S8). Carboxymethylation of Cys60 results in a peptide with three free carboxylic acid functions and carboxymethylation of Asp61 results in only two free carboxylic acid functions. Therefore, treatment of the purified C_{60}-K_{66}CM peptide with ethanol and propanol in the presence of concentrated H_2SO_4 should result in esterification of the free carboxylic acid residues on the peptide through a traditional Fisher esterification mechanism.[50] The *m*/*z* values of the mono- and di-esterified C_{60}-K_{66}CM peptide would be the same if either Cys60 or Asp61 were carboxymethylated (Table S3). The *m*/*z* values for the peaks eluting at 87 and 95 min (Fig. 5, Table S3) for the ethanol and propanol esterification, respectively, are consistent with the tri-esterified C_{60}-K_{66}CM peptide, which could only arise if the carboxymethylation occurs on Cys60. The MS/MS data presented in Figure 4 and the esterification data presented in Figure 5 confirms that Cys60 is the site of covalent modification between GLO1 and 4BAB.

5. Conclusion

This is the first reported inhibitor, 4BAB, that covalently modifies an amino acid residue near the active site of GLO1. This inhibitor specifically modifies Cys60 located in the hydrophobic

binding pocket adjacent to the GLO1 active site; however, this modification results in complete inactivation of only one active site, although leading to impaired catalytic efficiency at the second active site. We acknowledge that our inhibitor 4BAB is a lead molecule and as it is will have limited use pharmacologically. Rather our goal was a proof of principle that GLO1 could be inhibited covalently ay Cys60 within the enzyme active site. While we have not tested the specificity of 4BAB to GLO1 this molecule may serve as a template for the development of new GLO1 inhibitors that may combine this strategy with ones already reported for high affinity GLO1 inhibitors, potentially improving potency and specificity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.04.055.

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